

Environmental Agents That Have the Potential to Trigger Massive Apoptotic Neurodegeneration in the Developing Brain

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We review recent findings pertaining to several environmental agents (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, benzodiazepines, halothane, isoflurane, and propofol) that have the potential to delete large numbers of neurons from the developing brain by a newly discovered mechanism involving interference in the action of neurotransmitters [glutamate and γ -amino butyric acid (GABA) at *N*-methyl-D-aspartate (NMDA)] and GABA_A receptors during the synaptogenesis period, also known as the brain growth-spurt period. Transient interference (lasting ≥ 4 hr) in the activity of these transmitters during the synaptogenesis period (the last trimester of pregnancy and the first several years after birth in humans) causes millions of developing neurons to commit suicide (die by apoptosis). Many of these agents are drugs of abuse (ethanol is a prime example) to which the human fetal brain may be exposed during the third trimester by drug-abusing mothers. Ethanol triggers massive apoptotic neurodegeneration in the developing brain by interfering with both the NMDA and GABA_A receptor systems, and this can explain the reduced brain mass and lifelong neurobehavioral disturbances associated with intrauterine exposure of the human fetus to ethanol (fetal alcohol syndrome). Exposure of the immature brain in a medical treatment context is also of concern because many of these agents are drugs used frequently as sedatives, tranquilizers, anticonvulsants, or anesthetics in pediatric and/or obstetrical medicine. Because this is a newly discovered mechanism, further research will be required to fully ascertain the nature and degree of risk posed by exposure of the developing human brain to environmental agents that act by this mechanism. **Key words:** anesthetics, apoptosis, barbiturates, benzodiazepines, ethanol, GABA_A receptors, ketamine, NMDA receptors, phencyclidine, synaptogenesis. — *Environ Health Perspect* 108(suppl 3):383–388 (2000). <http://ehpnet1.niehs.nih.gov/docs/2000/suppl-3/383-388olney/abstract.html>

We are exploring mechanisms by which environmental agents can disrupt development of the brain, thereby giving rise to neurobehavioral disturbances that may be expressed either in childhood or with delayed onset in adulthood. Our interest in this topic was originally prompted by the discovery (1) three decades ago that glutamate (Glu), a widely used food additive, destroys neurons in the developing brain. Either oral or subcutaneous administration of Glu to infant animals (mice, rats, rabbits, guinea pigs, and monkeys) at doses that cause no obvious discomfort or disruption of normal behaviors, such as suckling, quietly destroys many neurons in the developing hypothalamus. Although this pathological event in the brain produces no overt signs of dysfunction during infancy, as the animals became adolescents they begin to display an abnormal body habitus (short and fat), and develop various disturbances in neuroendocrine function, including hypogonadism, infertility, and reduced pituitary gland size. The explanation for these abnormalities is that the hypothalamic neurons deleted during infancy perform important neuroendocrine regulatory functions pertaining to aspects of growth and development, including reproductive capacity, that mature and become functional in adolescence and early

adulthood (2). If Glu can induce lesions in the developing brain that remain silent until adolescence or early adulthood, other environmental agents may also be able to inflict silent damage on the developing brain; this damage may not be expressed as a functional abnormality until a later stage in ontogenesis, when the damaged brain circuitry fails to mature and assume its adult functions.

Our search for other environmental agents and other mechanisms that can subtly disrupt normal brain development and give rise to late onset neuropsychiatric disturbances has been fruitful. In this paper we review some of the more interesting results of this search.

Historical Perspective

The original observation that exogenous Glu damages the developing brain by exciting neurons to death (1) spawned a new field of research called excitotoxicology. A series of new findings in this field (3–5) led to the realization that endogenous Glu, by an excitotoxic mechanism, plays a major role in triggering neuronal degeneration in many if not all acute brain injury syndromes [e.g., stroke, perinatal asphyxia (hypoxia/ischemia), epilepsy, hypoglycemia, and head and spinal cord trauma]. This suggests the possibility that something as simple as compression of

the umbilical cord causing transient ischemia of the fetal brain could trigger excessive release of endogenous Glu and excitotoxic degeneration of large numbers of developing neurons. Although this is of considerable interest relative to the potential origins of neurodevelopmental disabilities, it is not an environmental mechanism. However, drugs that were developed as antiexcitotoxic neuroprotective agents fit broadly into the category of environmental agents, and some of these agents had interesting neurotoxic side effects. Examining the neurotoxic side effects of drugs that were developed as antagonists of *N*-methyl-D-aspartate (NMDA) Glu receptors provides a good starting point for our review.

NMDA Antagonist Neurotoxicity in the Adult Brain

Research pertaining to the neurotoxicity of NMDA antagonists dates back 10 years to the discovery (6) that systemic administration of drugs that block NMDA Glu receptors causes acute pathomorphological changes in cerebrocortical neurons of the adult rat brain. Low doses of NMDA antagonists, such as MK801 and phencyclidine (PCP), produced reversible pathomorphological changes in cerebrocortical neurons, whereas higher doses of these agents induced irreversible neuronal degeneration that initially was thought to be limited to the posterior cingulate and retrosplenial cortices (6,7). However, more recent findings document that high doses of these agents cause irreversible degeneration of neurons not only in the cingulate and retrosplenial cortices but in a number of other corticolimbic brain regions (8–11).

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action was highly receptor specific in that many agents that act at other receptor subtypes or other transmitter receptor systems were tested and had no effect on MK801 neurotoxicity.

release of excitatory transmitters, both acetylcholine (ACh) and Glu, in the cerebral cortex. By direct injection of agents into the brain, coupled with microdialysis methods (20,28–30), additional aspects of the circuitry depicted in Figure 1 have been confirmed, whereas other aspects remain to be studied.

Our early studies pertaining to NMDA antagonist neurotoxicity focused exclusively on the adult central nervous system (CNS), with the exception of one study (31) in which we administered NMDA antagonists (MK801 and PCP) to rats at various stages of development (fetal, infancy, adolescence, and adulthood) and we examined the brains 4 hr later for evidence of acute pathomorphological changes in cerebrocortical neurons. We found that very high doses of these NMDA antagonists do not trigger acute changes in cerebrocortical neurons of immature rats (fetal, infant, and early adolescent).

Distinguishing Excitotoxic from Apoptotic Neuronal Cell Death

The diagram illustrates the basal forebrain circuitry. At the top, a **PC/RS neuron** is shown with its cell body and dendrites. It receives input from **m₃** (muscarinic receptor 3) and σ (opioid receptor). The neuron's axon terminals release **Glu** (glutamate) and **GABA** (gamma-aminobutyric acid). The **Glu** terminals are labeled **Glu (non-NMDA)** and **Glu**. The **GABA** terminals are labeled **GABA** and **NMDA**. The **Glu** terminals have excitatory connections (+) to the **Anterior thalamus** and **Basal forebrain**. The **GABA** terminals have inhibitory connections (-) to the **Anterior thalamus** and **Basal forebrain**. The **Anterior thalamus** has excitatory connections (+) to the **Basal forebrain** and **5HT_{2A}** (5-hydroxytryptamine 2A). The **Basal forebrain** has excitatory connections (+) to the **5HT_{2A}** and **5HT** (5-hydroxytryptamine). The **5HT_{2A}** has excitatory connections (+) to the **Basal forebrain** and **NE** (norepinephrine). The **5HT** has excitatory connections (+) to the **Basal forebrain** and **NE**. The **NE** has excitatory connections (+) to the **Basal forebrain** and **5HT**. The **Basal forebrain** also has inhibitory connections (-) to the **Anterior thalamus** and **5HT_{2A}**. The **Anterior thalamus** has inhibitory connections (-) to the **Basal forebrain** and **5HT_{2A}**. The **5HT_{2A}** has inhibitory connections (-) to the **Basal forebrain** and **5HT**. The **5HT** has inhibitory connections (-) to the **Basal forebrain** and **NE**. The **NE** has inhibitory connections (-) to the **Basal forebrain** and **5HT**. The **Basal forebrain** also has inhibitory connections (-) to the **Anterior thalamus** and **5HT_{2A}**. The **Anterior thalamus** has inhibitory connections (-) to the **Basal forebrain** and **5HT_{2A}**. The **5HT_{2A}** has inhibitory connections (-) to the **Basal forebrain** and **5HT**. The **5HT** has inhibitory connections (-) to the **Basal forebrain** and **NE**. The **NE** has inhibitory connections (-) to the **Basal forebrain** and **5HT**.

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identified as apoptosis is truly apoptosis. To explore this issue we performed a side-by-side ultrastructural comparison of a prototypic excitotoxic process (Glu-induced degeneration of neurons in the infant rat hypothalamus) and a prototypic apoptotic process, physiological cell death, the natural process by which biologically redundant neurons are deleted from the developing brain. In this work (33), we demonstrated by electron microscopy that the type and sequence of changes characterizing excitotoxic cell death are entirely different from those characterizing apoptotic cell death. However, DNA fragmentation tests (TUNEL stain or gel electrophoresis) did not distinguish between the two processes. These tests were positive for both excitotoxic and apoptotic cell death, signifying that both types of cell death degrade DNA into similar fragments that are detected by these methods, but because the two cell death processes are clearly dissimilar by ultrastructural analysis, one must conclude that DNA fragmentation analysis is not a valid or reliable test for diagnosing apoptosis. Our finding that DNA fragmentation tests are not specific for apoptosis and do not reliably distinguish apoptosis from excitotoxic cell death is consistent with similar findings from several other laboratories (34–36), including a study from the Kerr laboratory (37) [Kerr originally coined the term “apoptosis” (38)].

The Potential of NMDA Antagonists to Induce Apoptotic Neurodegeneration in the Developing Brain

We began additional studies aimed at determining whether other examples of excitotoxic neurodegeneration in the *in vivo* mammalian brain could be distinguished from apoptosis. The first example studied was a new model that we developed for investigating concussive head trauma in infant rats. Using this model, we found that a concussive force applied to the skull overlying the parietal cortex of the 7-day-old rat caused a relatively small excitotoxic lesion at the impact site that evolved rapidly to end-stage neuronal necrosis within 4 hr after impact. Administration of MK801 before concussive injury protected against this acute excitotoxic lesion at the impact site (39). We then discovered that over the ensuing 24 hr, additional foci of neurodegeneration developed at distant sites and the degenerating neurons at these sites qualified for a diagnosis of apoptosis by ultrastructural criteria (40). We applied various neuroprotective strategies and were surprised to find that MK801 not only failed to protect against the delayed, distant, apoptotic response, it increased the magnitude of this response (41).

These findings raised an interesting question: because NMDA antagonists promote the apoptotic neurodegenerative process induced in the immature brain by head trauma, is it possible that they might also promote the spontaneous apoptotic neurodegenerative process that occurs naturally (independent of head trauma) in the normal developing brain? We investigated this and found that indeed MK801, when administered to 7-day-old infant rats, triggers a massive apoptotic neurodegenerative response affecting many neurons in several major regions of the developing brain (42). In addition, we administered PCP and ketamine to 7-day-old infant rats by dosing regimens calculated to keep the rat pups intoxicated for up to 8 hr and found that both of these NMDA antagonists trigger a robust neurodegenerative response in the developing brain (42).

In additional experiments, we determined that the time window of vulnerability to the apoptosis-inducing action of NMDA antagonists coincides with the period of synaptogenesis, also known as the brain growth spurt period. This period in the rat is largely confined to the postnatal period; it begins 1 day before birth and terminates at 10–14 days after birth, whereas in the human it spans the last 3 months of pregnancy and extends into the first several years postnatally (43). In these experiments we also observed that within the brain growth-spurt period, different neuronal populations become sensitive at different times to the mechanism by which NMDA antagonists trigger apoptotic degeneration (42). Thus, depending on whether exposure occurs in the early, mid, or late stage of the brain growth-spurt period, different combinations of neuronal groups will be deleted from the brain, from which it follows that this neurodevelopmental mechanism has the potential to produce a variety of neurobehavioral deficit syndromes.

The Potential of Ethanol and GABAergic Agents to Induce Apoptotic Neurodegeneration in the Developing Brain

Evidence that ethanol has NMDA antagonist properties (44–47) prompted us to evaluate its ability to mimic the proapoptotic effects of other NMDA antagonists. Administration of ethanol to 7-day-old infant rats revealed that it triggers a neurodegenerative response that is even more robust than the response to MK801 (Figure 2) (48,49). Evaluation of the ethanol-induced degenerative response by electron microscopy revealed that it conforms to the criteria for apoptotic cell death (Figure 3). The window of vulnerability to ethanol-induced apoptosis was the same as that for MK801 (coincides with the synaptogenesis/brain

growth-spurt period). In addition, we found that within the brain growth spurt period different neuronal populations become sensitive at different times to the mechanism by which ethanol triggers apoptotic degeneration. We also determined that the minimum condition for triggering neurodegeneration was maintaining blood ethanol concentrations ≥ 200 mg/dL for 4 consecutive hr, and that if ethanol concentrations remained > 200 mg/dL for > 4 hr, the degenerative response became progressively more severe and more widespread in proportion to how long the concentrations remained above this level.

Because ethanol triggered apoptosis in some brain regions not typically affected by NMDA antagonists, we attempted to identify other possible mechanisms to explain ethanol's effects in these brain regions. We were unable to demonstrate an appreciable apoptotic response to agents that act as either agonists or antagonists at dopamine receptors; or that block kainic acid, muscarinic cholinergic receptors, or block voltage gated ion channels; but a robust apoptotic response was triggered by agents (benzodiazepines and barbiturates) that mimic or potentiate the action of GABA at GABA_A receptors. We tested diazepam (10–30 mg/kg ip at 0 hr, $n = 6$), clonazepam (0.5–4 mg/kg at 0 hr, $n = 6$), pentobarbital (10 mg/kg ip at 0 and 4 hr, $n = 6$), and phenobarbital (50–75 mg/kg ip at 0 hr, $n = 6$). These agents triggered widespread cell death in the infant rat brain in a dose-dependent manner; cell death, by ultrastructural analysis, was apoptotic. The pattern of degeneration was similar for each GABAergic agent but this pattern differed in several major respects from that induced by NMDA antagonists (Figure 2). However, superimposing one pattern on the other resulted in a composite pattern closely resembling that induced by ethanol (Figure 2).

Neurotoxic versus Neuroprotective Effects of Ethanol in the Immature versus Adult Brain

Evidence that ethanol has neurotoxic effects on the immature brain that appear to be mediated in part by the NMDA antagonist properties of ethanol, and the knowledge that NMDA antagonists also have neurotoxic effects in the adult brain raises the interesting question whether ethanol produces the NMDA antagonist type of neurotoxicity in the adult brain. To evaluate this we administered large doses of ethanol to adult rats and found no evidence for the neurotoxic reaction that NMDA antagonists cause in the adult cerebral cortex. We reasoned that failure of ethanol to trigger a neurotoxic reaction might imply that ethanol's potential to produce neurotoxicity by blocking NMDA receptors

is counteracted by some other property of ethanol such as its action as a positive modulator of GABA_A receptors. If this were the case, and if its action at GABA receptors were particularly strong, it might be possible to show that ethanol, in addition to being able to protect against its own NMDA antagonist

neurotoxic potential, can protect against the neurotoxic potential of other NMDA antagonists. To test this we administered ethanol together with a neurotoxic dose of MK801 and found that ethanol in the adult brain provides strong protection against the neurotoxic action of MK801 (50).

NMDA Antagonist Properties of Nitrous Oxide (Laughing Gas)

Nitrous oxide (laughing gas) is a general anesthetic agent that has been used in dentistry and medicine for over a century, during which time little insight into its mechanism of action has been gained. Recently, we discovered that nitrous oxide, in clinically relevant concentrations, has all of the properties of an NMDA antagonist. It protects neurons in the rat hypothalamus against the excitotoxic action of systemically administered NMDA, it mimics PCP and MK801 in injuring or killing neurons in the adult rat cerebral cortex, and in patch clamp electrophysiological experiments it blocks currents induced in hippocampal neurons by NMDA. In addition, its pharmacological profile as a human anesthetic agent parallels that of ketamine, a well known NMDA antagonist. These findings were published recently (51), as was a follow-up study detailing the electrophysiological profile of nitrous oxide as an NMDA antagonist (52).

Significance of These Findings in an Environmental Health Context

In part, the public health significance of our findings stems from the fact that ethanol is, and has been for several thousand years, the most widely abused drug in the world. Over the millennia, ethanol has caused, and continues to cause, more neurodevelopmental morbidity in human offspring than any other agent in the human environment. Cases of severe impairment (the tip of the iceberg) are referred to as fetal alcohol syndrome (FAS) and less severe impairment (the iceberg itself) as fetal alcohol effects (FAE). The human synaptogenesis/brain growth-spurt period includes the last 3 months of gestation (43) and the blood ethanol levels required to trigger apoptotic neurodegeneration in the immature rat brain (200 mg/dL lasting ≥ 4 hr) are in the range that a third-trimester human fetus might be exposed to by a pregnant mother who imbibes ethanolic beverages for several hours in a single drinking episode. Our finding that transient exposure of the *in vivo* mammalian brain to ethanol during the synaptogenesis period causes neurons to commit suicide by the tens of millions provides a likely explanation for the reduced brain mass and neurobehavioral disturbances associated with the human FAE/FAS. What types of neurobehavioral disturbances might be expected from this mechanism? Hyperactivity/attention deficit disorder and learning disability ranging in severity from mild impairment to mental

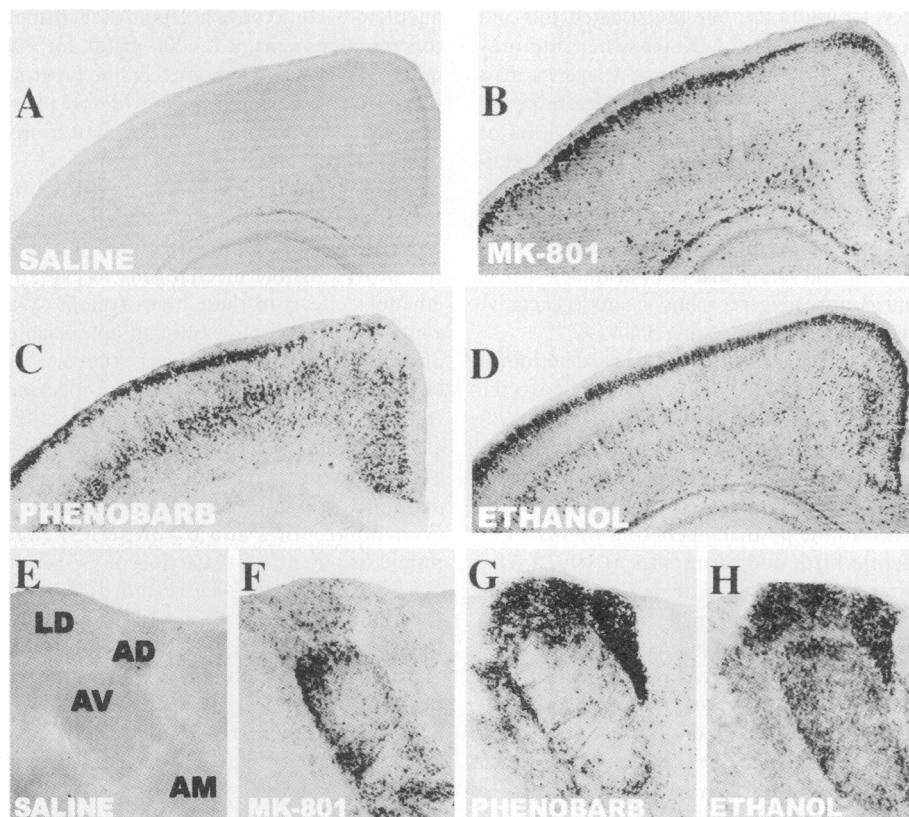


Figure 2. (A–D) Low magnification (17.5 \times) light microscopic overviews of silver-stained transverse sections from the parietal and cingulate cortex of 8-day-old rats treated 24 hr previously with saline, MK-801 (NMDA antagonist), phenobarbital, or ethanol. Abbreviations: AD, anterodorsal; AM, anteromedial; AV, anteroventral; LD, laterodorsal. Degenerating neurons (small dark dots) are abundantly present in several brain regions after MK-801, phenobarbital, or ethanol treatment but are only sparsely present after saline treatment. MK-801 and phenobarbital both affect neurons superficial to the cortical surface, whereas the middle cortical layers are affected prominently by phenobarbital and are relatively spared by MK-801. The ethanol pattern resembles a combination of the MK-801 and phenobarbital patterns. (E–H) Light micrographs (38.5 \times) depicting the anterior thalamus at the level of the LD, AD, AV, and AM nuclei. MK-801 affects the LD, AV, and AM nuclei but not the AD nucleus, and phenobarbital prominently affects the LD and AD but almost entirely spares the AV and AM nuclei. The ethanol pattern includes all four nuclei, as would be expected if it acts by a combined action involving the blockade of NMDA receptors plus the activation of GABA_A receptors. Adapted from Ikonomidou et al. (49).

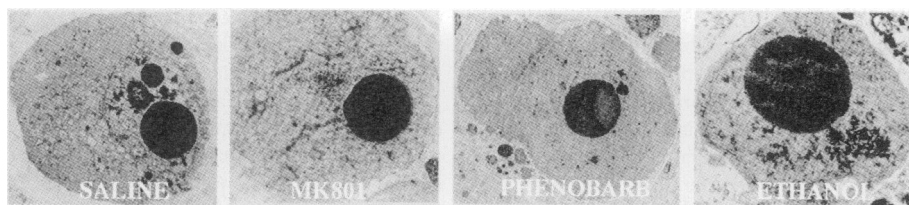


Figure 3. Electron micrographs (1,350 \times) illustrating that apoptotic neurodegeneration induced by MK801, phenobarbital, or ethanol has the same ultrastructural appearance as physiological cell death, the natural cell death process sparsely present in saline controls. As we recently described (31,40), in both spontaneous and induced apoptosis the earliest signs are the formation of spherical chromatin masses and flocculent densities in the nucleus while the nuclear envelope remains intact and cytoplasmic organelles are relatively unaltered. This is followed in the mid and late stages by fragmentation of the nuclear envelope, intermixing of nucleoplasmic and cytoplasmic contents, and progressive condensation of the entire cell. All four examples have a similar appearance because they are all in the mid stage of apoptotic neurodegeneration. Adapted from Ikonomidou et al. (49).

retardation are the types of FAE/FAS disturbance that have received the most attention. However, Famy et al. (53) recently studied a sizeable cohort of adult subjects who received a FAE/FAS diagnosis in childhood and found that a high percentage (72%) required psychiatric attention as adults for a variety of adult-onset psychiatric problems, including a 40% incidence of psychosis and 44% incidence of major depressive disorder. Thus, ethanol is a prime example of an environmental agent that can quietly delete large numbers of neurons from the developing brain by an important mechanism that we are just beginning to understand. During a critical stage in development (brain growth-spurt/synaptogenesis period) when neurons are rapidly expanding their dendritic trees and establishing trillions of synaptic connections every day, ethanol interferes with transmitter activity at two of the most ubiquitous transmitter receptor systems in the developing brain—the NMDA Glu and GABA_A receptor systems. As a result of this interference, developing neurons apparently receive a message that developmental events are not progressing normally, which is interpreted as a signal to commit suicide. Of considerable interest is our finding that within the brain growth-spurt period different neuronal populations have different temporal patterns for responding to the apoptosis-inducing effects of ethanol. Thus, depending on the timing of exposure, different combinations of neuronal groups will be deleted, which explains why fetal ethanol exposure gives rise to a wide spectrum of neuropsychiatric disturbances (53). In other recent writings (54) we discussed in greater detail the possibility that certain patterns of neuronal or receptor losses might be more conducive to the subsequent development of a psychotic illness such as schizophrenia, whereas other patterns might be more conducive to nonpsychotic psychiatric illnesses.

Because ethanol is both an NMDA antagonist and potentiator of GABA_A transmission, it would be expected to have a compound neurotoxic action in the developing brain (mediated simultaneously through both NMDA and GABA_A receptors). However, in the adult brain ethanol may be much less toxic because the neurotoxic potential of its NMDA receptor-blocking activity will be counteracted and cancelled out by its GABA_A-potentiating activity. Our evidence that ethanol triggers severe apoptotic neurodegeneration in the developing brain (49), but is relatively nontoxic or even neuroprotective in the adult brain (50), supports this interpretation. Our evidence is also consistent with the historical reality that over the millennia ethanol has served as the inebriant and euphoriant of choice for human adults who

assumed that its effects are totally reversible and harmless, an assumption that has resulted in countless human fetuses being born with myriad neurobehavioral disturbances ranging from hyperactivity/attention-deficit and learning disorders to major adult-onset disturbances including depression and psychosis (53).

To fully appreciate the public health significance of our findings, it must be recognized that in addition to risk pertaining to ethanol, both NMDA antagonists and GABA_A potentiators are drugs of abuse and also are drugs used frequently as sedatives, tranquilizers, anticonvulsants, or anesthetics in pediatric and/or obstetrical medicine. Because the human brain growth spurt spans not only the last trimester of pregnancy but several years after birth (41), the developing human brain may be exposed to these agents illicitly by drug-abusing pregnant mothers or licitly by the medical profession. For example, both ketamine and nitrous oxide are used frequently in pediatric anesthesia and are commonly administered as an anesthetic cocktail together with various agents (barbiturates, benzodiazepines, isoflurane, and propofol) that act as positive modulators of GABA_A receptors. Thus, studies are needed to clarify whether exposure of the immature brain to cocktails containing combinations of NMDA antagonists and GABA_A potentiators during the last half of the brain growth-spurt period poses a risk of silently deleting large numbers of neurons from the developing brain.

Summary

In this paper we have summarized recent findings pertaining to several environmental agents (ethanol, phencyclidine, ketamine, nitrous oxide, barbiturates, benzodiazepines, halothane, isoflurane, and propofol) that have the potential to delete large numbers of neurons from the developing brain by a newly discovered mechanism involving interference in the action of neurotransmitters (Glu and GABA) at NMDA and GABA_A receptors during the synaptogenesis period, also known as the brain growth-spurt period. Interference in the activity of these transmitters during the synaptogenesis period (the last trimester of pregnancy and the first several years after birth in humans) causes developing neurons to commit suicide (die by apoptosis). Many of these agents are drugs of abuse to which the human fetal brain may be exposed during the third trimester by drug-abusing mothers. Ethanol, the most widely abused drug in the world, triggers massive apoptotic neurodegeneration in the developing brain by interfering with both the NMDA and GABA_A receptor systems. This is a likely explanation for the reduced brain mass and lifelong neurobehavioral disturbances resulting from intrauterine exposure of the human fetus to ethanol (FAS).

Exposure of the immature brain in a medical treatment context is also of concern because many of these agents are drugs used frequently as sedatives, tranquilizers, anticonvulsants, or anesthetics in pediatric and/or obstetrical medicine. Because this is a newly discovered mechanism, further research will be required to develop a full appreciation for the nature and degree of risk posed by exposure of the developing human brain to environmental agents that act by this mechanism.

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